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Morpho-histological study of somatic embryo-like structures in hypocotyl cultures of *Pelargonium × hortorum* Bailey

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Abstract Somatic embryo-like structures were produced from the hypocotyls of ten cultivars of *Pelargonium × hortorum* using the protocols of Marsolais et al. (1991; Can J Bot 69:1188–1193) and Slimmon et al. (1991; Plant Cell Rep 10:587–589) and their embryonic natures evaluated. Nine cultivars responded, and 937 structures were formed. Regeneration corresponded well with published data. The somatic embryo-like structures were globular- to leaf-shaped or similar to shoots. A root pole was never visible. Histological examinations confirmed the lack of bipolarity and revealed vascular connections to the explant in the more developed structures. Therefore, these structures cannot be classified as somatic embryos. The importance of these results is discussed in terms of evaluating published protocols for the propagation of these pelargoniums by somatic embryogenesis from hypocotyls.

Keywords *Pelargonium × hortorum* · Somatic embryogenesis · Organogenesis · Histology

Introduction

Pelargoniums are among the world's most popular ornamental plants. Mass propagation is from cuttings or seed (Berninger 1993), which is very expensive. The use of cuttings involves problems with several bacterial and viral diseases (Daughtrey and Wick 1993; Nameth and Adkins 1993), and propagation from seed is only practical for cultivars bred for this purpose (Craig 1993; Lange and Horn 1996). The application of shoot-tip culture for large-

scale propagation is unsuitable because the multiplication rates are very low (Appelgren et al. 1991). Regeneration via somatic embryogenesis has the potential to facilitate more efficient propagation techniques.

The most desirable explants for such purposes are those that are available from adult plants following quality testing. In *Pelargonium × hortorum*, the embryogenic response of those explants is very low. Average regeneration rates of up to 0.1 to 0.5 somatic embryos per explant have been achieved using petioles (Marsolais et al. 1991; Haensch 1999). Therefore, most of the published work on somatic embryogenesis in *Pelargonium × hortorum* is based on explants of very young seedlings, especially hypocotyls (Marsolais et al. 1991; Slimmon et al. 1991; Visser et al. 1992; Qureshi and Saxena 1992; Gill et al. 1993, 1994; Hutchinson and Saxena 1996a, 1996b; Hutchinson et al. 1996a, 1996b, 1997a, 1997b, 2000; Murthy et al. 1996, 1999; Wilson et al. 1996; Croke and Cassels 1997; Senaratna et al. 1999, 2002; Madakadze and Senaratna 2000; Madakadze et al. 2000; Murch and Saxena 2001). Somatic embryos are defined, with general acceptance (Brown et al. 1995), as new individuals that develop both a shoot apex and a discrete radicular end (Haccius 1978). The embryo-like structures from hypocotyl cultures of *Pelargonium × hortorum* have been classified as somatic embryos, although no distinct and unambiguous root pole could be demonstrated (Marsolais et al. 1991; Visser et al. 1992; Qureshi and Saxena 1992; Gill et al. 1993, 1994; Hutchinson and Saxena 1996b; Murthy et al. 1996; Croke and Cassels 1997). The uncertainty in the classification of these regenerants is suggested by the fact that even Croke and Cassels (1997) used terms such as “putative somatic embryos” and “somatic embryo-like structures”. Histological examinations were rarely made, and when they were, the classification of the regenerating structures as somatic embryos was unconvincing insofar as the more advanced stages did not appear to be clearly separated from the maternal explant, and their bipolarity was not verified (Gill et al. 1993; Hutchinson et al. 1996a). The aim of the investigation reported here was, therefore, to

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produce embryo-like structures on the hypocotyls of *Pelargonium × hortorum* using two published protocols (Marsolais et al. 1991; Slimmon et al. 1991) and to check the embryonic nature of these structures.

Materials and methods

In vitro regeneration

Hypocotyl cultures were established according to the methods of Marsolais et al. (1991) or Slimmon et al. (1991) with minor modifications. An assortment of ten cultivars of *Pelargonium × hortorum* were studied, two of which, White Orbit and Violet Orbit, had also been used by Marsolais et al. (1991) (Table 1). Seeds were surface-sterilized by shaking for 20 min in a 1.4% solution of sodium hypochlorite and rinsing five times in sterile distilled water. Each seed was incubated on distilled water solidified with 6.5 g/l agar, at 22°C, in the dark, in a 50-ml test tube sealed with plastic film. After 4–5 days, the hypocotyls of the germinated plants reached lengths of 1.0–2.5 cm. At this time, the hypocotyls were cut into 0.5-cm-long segments. The segments were placed on 25 ml medium in 90×15-mm petri dishes that were subsequently wrapped with plastic film and incubated in a growth chamber at 24°C under a 16/8-h (day/night) photoperiod (white fluorescent light supplied at an intensity of 46 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Two different media were compared: (1) GCM medium supplemented with 1 $\mu\text{mol/l}$ indole-3-acetic acid (IAA), 2 $\mu\text{mol/l}$ 6-benzylaminopurine (BAP), 30 g/l sucrose, and 6.5 g/l agar (Serva, Heidelberg, Germany), pH 5.6 (Marsolais et al. 1991); (2) MS (Murashige-Skoog 1962) medium supplemented with 1 $\mu\text{mol/l}$ IAA, 8 $\mu\text{mol/l}$ BAP, 30 g/l sucrose, and 2.5 g/l Gelrite (Duchefa, Haarlem, The Netherlands), pH 5.0 (Slimmon et al. 1991). In each medium, iron was provided as ethylenediaminetetraacetic acid iron salt. In the MS medium, the iron content was lowered to 86.9 μM . Each petri dish contained ten explants and the treatments were replicated four times. After 28 days, the cultures were assessed for the number and kinds of structures formed by means of a stereomicroscope.

Histological evaluation

Thirty-one structures derived from cvs. White Orbit and Scarlet Orbit were evaluated histologically. All of these were formed on MS medium as modified by Slimmon et al. (1991).

Explants with regenerated structures were fixed in a solution of formalin, alcohol and acetic acid (FAA), 100 ml of which contains 5.4 ml formalin (37%), 65.6 ml ethanol (96%), 5 ml glacial acetic

acid, and 24 ml distilled water (Gerlach 1984). Parts of the explants with representative structures were cut and embedded in hydroxyethylmethacrylate (Histo-Technique-Set Technovit 7100; Kulzer, Wehrheim, Germany). At the beginning of this process, the specimens were dehydrated in 2-h steps through a graded series of ethanol (70%, 90%, 96%, and 100%). The samples were then pre-infiltrated overnight with a mixture of equal parts of 100% ethanol and Technovit 7100 base liquid. The explants were then transferred into an infiltration solution of 100 ml Technovit 7100 base liquid and 1 g hardener I for 1 day. A vacuum was established for 30 min at the start of the last two processes. Explants were embedded in Teflon moulds with a mixture of 15 parts infiltration solution and one part hardener II. The samples were polymerized for 1 h at room temperature and for a further 6 h at 37°C. Specimens were mounted on block-holders with Technovit 3040. Slices (6 μm) were cut at room temperature using a Jung CM 1800 microtome equipped with type 818 disposable microtome blades (both from Leica Instruments, Nussloch, Germany). Slices were stretched on a bath of distilled water and mounted on slides. They were then stained with 0.05% toluidine blue O (Serva, Heidelberg, Germany) dissolved in 1% sodium tetraborate decahydrate buffer (Hutchinson et al. 1996a), rinsed in distilled water, dried, and covered with Entellan (Merck, Darmstadt, Germany) and a cover slip. This procedure stains the cytoplasm and un lignified cell walls red and the DNA-containing structures and lignified cell walls blue (Gerlach 1984). Microscopic analysis was performed using a Leitz DMR microscope (Leica, Wetzlar, Germany) with a Wild MPS 48/52 camera (Leica, Heerbrugg, Switzerland).

Results

In vitro regeneration

Regenerating structures were observed on the hypocotyl explants of nearly all cultivars. These structures occurred on the epidermis of the long sides of the explants and were globular- to leaf-shaped or similar to shoots, greenish, and partly covered with hairs (Fig. 1). No root pole was visible. An unambiguous classification of these structures was not possible without histological evaluation. Therefore, they were designated “somatic embryo-like structures” for the interim. In total, 937 structures were regenerated from 675 surviving explants. Cultivar White Orbit showed the highest response on GCM and

Table 1 Formation of somatic embryo-like structures on the hypocotyl segments of several cultivars of *Pelargonium × hortorum* grown on different media

Cultivar	GCM medium with 1 $\mu\text{mol/l}$ IAA, 2 $\mu\text{mol/l}$ BAP, 6.5 g/l agar, pH 5.6			MS medium with 1 $\mu\text{mol/l}$ IAA, 8 $\mu\text{mol/l}$ BAP, 2.5 g/l Gelrite, pH 5.0		
	Number of surviving explants	Number of somatic embryo-like structures per surviving explant		Number of surviving explants	Number of somatic embryo-like structures per surviving explant	
		Mean	Maximum		Mean	Maximum
White Orbit	40	0.65	4	30	7.73	21
Multibloom Scharlach	34	0.00	0	33	7.18	19
Pink Orbit	36	0.19	3	36	6.03	34
Elite Scarlet	40	0.05	1	40	2.60	11
Scarlet Orbit	40	0.13	1	40	1.93	8
PAC Adretta	37	0.00	0	17	0.47	3
Violet Orbit	40	0.00	0	40	0.33	5
PAC Quix	39	0.00	0	34	0.18	5
PAC Libelle	23	0.04	1	14	0.14	1
PAC Sitta	38	0.00	0	24	0.00	0

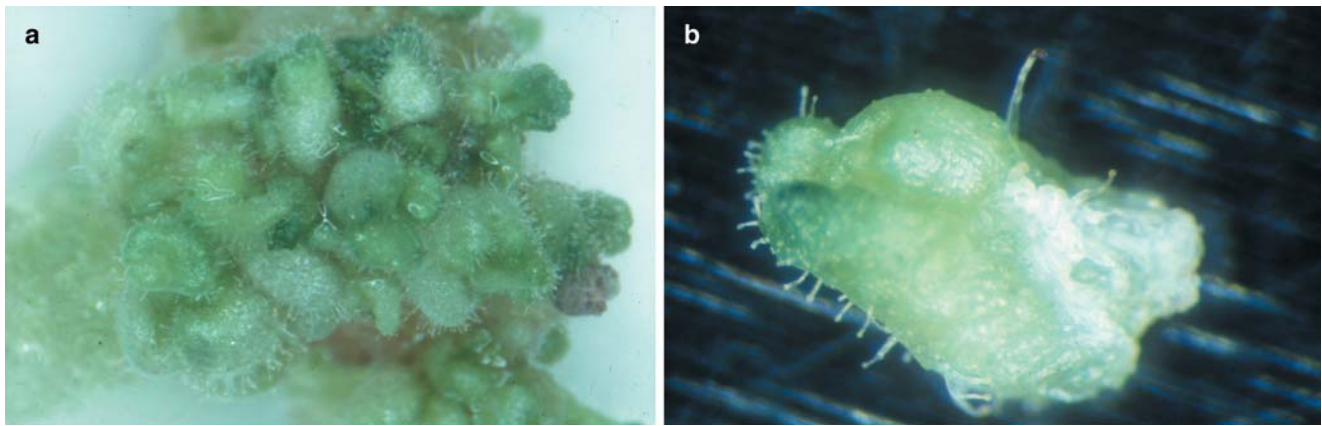


Fig. 1a, b Regeneration in cultures of hypocotyls of *Pelargonium* × *hortorum* Multibloom Scharlach. **a** Group of somatic embryo-like structures (×6). **b** Single detached somatic embryo-like structure (×23)

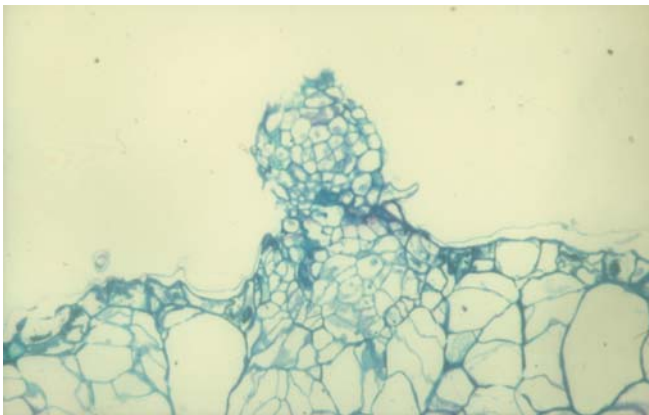


Fig. 2 Longitudinal section of a globular somatic embryo-like structure on a hypocotyl segment of *Pelargonium* × *hortorum* Scarlet Orbit (×183)

MS media, with mean somatic embryo-like structures per surviving explant of 0.65 and 7.73 respectively, whereas there was no regeneration of the PAC Sitta cultivar on either medium (Table 1). Cultivar Violet Orbit did not form somatic embryo-like structures on GCM medium and showed only a weak response on MS medium. Of the two media, the MS medium as modified by Slimmon et al. (1991) produced much better results in terms of the number of responsive genotypes and the number of regenerating structures.

Histological evaluation

Histological slices confirmed the initial globular shape of the regenerants (Fig. 2). No root pole could be demonstrated in the more developed structures (Fig. 3a). Although these structures could be assigned to the globular or heart stage on the basis of their appearance at the beginning of regeneration, the vast majority of their cells were parenchymatous, i.e., with a strongly vacuolized cytoplasm. In addition to the absence of a root

meristem, spiral vessels (i.e., elements of the xylem bundle) were visible. These vascular strands in the regenerating structures stretched to the parenchymal cells of the maternal explant (Fig. 3b) or even into the tissue of the explant (Fig. 3c, d).

Discussion

From the several publications that have reported on somatic embryogenesis in hypocotyl cultures of *Pelargonium* × *hortorum* (Marsolais et al. 1991; Slimmon et al. 1991; Visser et al. 1992; Qureshi and Saxena 1992; Gill et al. 1993, 1994; Hutchinson and Saxena 1996a, 1996b; Hutchinson et al. 1996a, 1996b, 1997a, 1997b, 2000; Murthy et al. 1996, 1999; Wilson et al. 1996; Croke and Cassels 1997; Senaratna et al. 1999, 2002; Madakadze and Senaratna 2000; Madakadze et al. 2000; Murch and Saxena 2001), we selected two reliable protocols to generate somatic embryo-like structures and to check their embryonic nature: (1) GCM medium supplemented with 1 μmol/l IAA and 2 μmol/l BAP, which was used to screen cultivars by Marsolais et al. (1991); (2) MS medium supplemented with 1 μmol/l IAA and 8 μmol/l BAP, which was used as a control by Slimmon et al. (1991).

It was possible to reproduce the regeneration of the structures that were classified as somatic embryos by Marsolais et al. (1991) and Slimmon et al. (1991). This result is supported by a correspondence in the morphological appearance of these structures (Fig. 1) as well as by the similarity of the quantitative responses (Table 1). Cultivar White Orbit responded best to both of the protocols used. This is consistent with the assessment of Marsolais et al. (1991), who evaluated this cultivar as the fourth best in terms of the number of somatic embryos produced from an assortment of 30 cultivars of *Pelargonium* × *hortorum*. Similarly, there was a correspondence between the lack of regeneration shown by cv. Violet

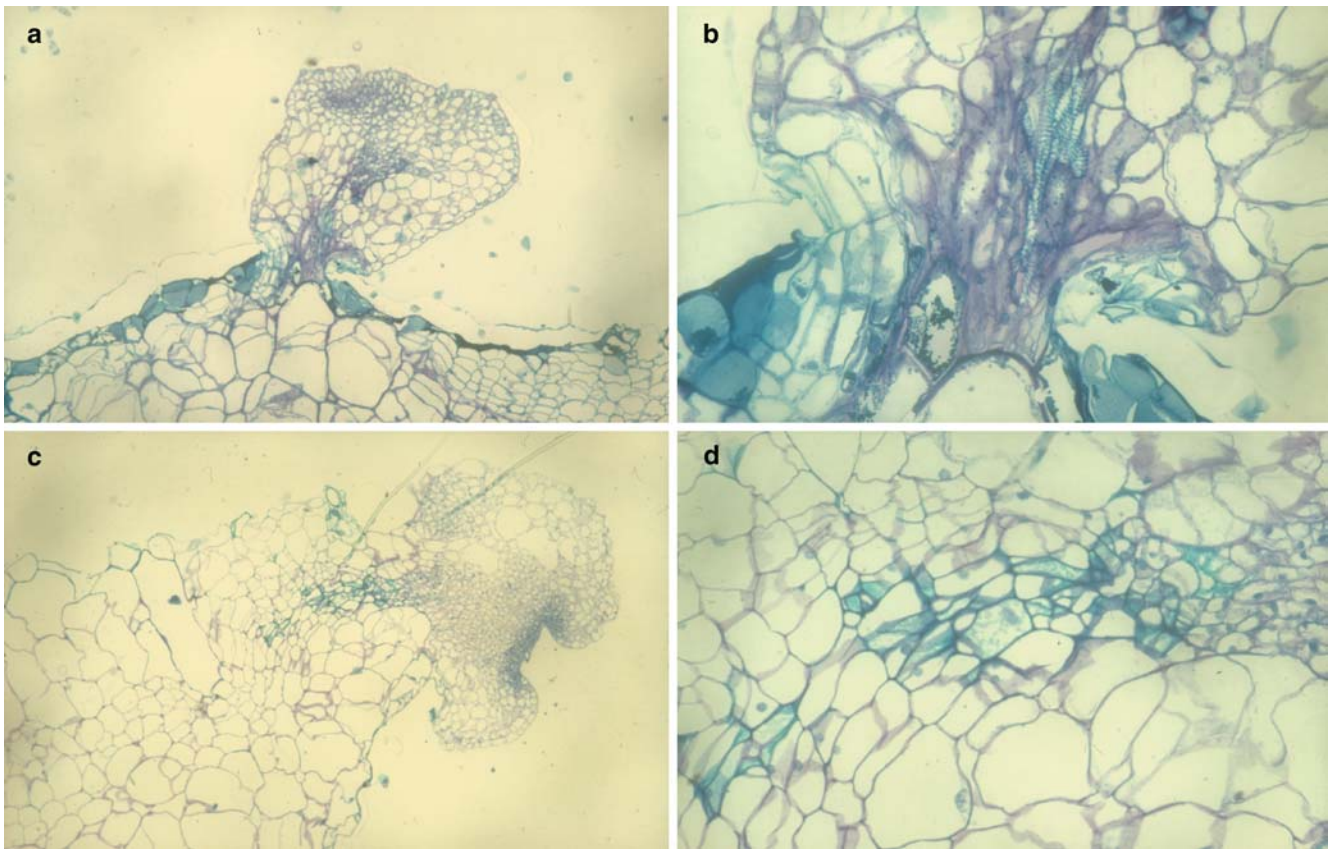


Fig. 3 Longitudinal sections of more developed somatic embryonic structures on hypocotyl segments of *Pelargonium × hortorum* Scarlet Orbit (**a, b**) and White Orbit (**c, d**). In each case, the tissue of the regenerant is predominantly parenchymatous, and there is no

root pole. **a** Vascular strands are present and extend to the explant tissue ($\times 92$), **b** Fig. 3a in detail, showing vascular strands ($\times 366$). **c** A vascular connection is established into the tissue of the explant ($\times 92$), **d** Fig. 3c in detail ($\times 366$)

Orbit on GCM medium in this study and its weak response in the study of Marsolais et al. (1991).

Although the regenerated structures superficially resembled somatic embryos, it was not possible to confirm their embryonic nature. Somatic embryos are individual bipolar structures with no vascular connection to the mother plant at any time of their life (Haccius 1978) and which develop from somatic cells through characteristic embryological stages (Williams and Maheswaran 1986). The regenerating structures in the present study did have a globular shape at the beginning of their development, but the majority of their cells were parenchymatous, i.e., vacuolized (Fig. 2). Globular somatic embryos normally consist of small cells with dense cytoplasm and large nuclei, as has been observed in other species (*Daucus*, Schiavone and Cooke 1985; *Nigella*, Banerjee and Gupta 1976; *Panicum*, Lu and Vasil 1985). More importantly, and in contradiction to the definition cited above, none of the 937 somatic embryo-like structures in the present experiment showed a root pole (Fig. 1). This lack of bipolarity was confirmed by histological examination. Furthermore, histology revealed vascular connections near or into the explant in the more developed structures (Fig. 3). On the basis of our findings, the structures regenerated on the hypocotyls of *Pelargonium × horto-*

rum on GCM medium supplemented with 1 $\mu\text{mol/l}$ IAA and 2 $\mu\text{mol/l}$ BAP (Marsolais et al. 1991) or on MS medium supplemented with 1 $\mu\text{mol/l}$ IAA and 8 $\mu\text{mol/l}$ BAP (Slimmon et al. 1991) cannot be classified as somatic embryos, predominantly because they are not bipolar. According to the criteria formulated by Haccius (1978), some of these regenerants are shoots or shoot-like structures because they have a shoot meristem and a vascular connection into the explant (Fig. 3c, d). Other regenerants can only be regarded as leaf-like structures because their tissue is parenchymatous, contains partially vascular strands, and has no shoot or root meristem (Figs. 2, 3a, b).

These results indicate that the published protocols for the propagation of *Pelargonium × hortorum* via somatic embryogenesis from hypocotyls must be evaluated critically. A resemblance between the external appearances of the regenerating structures and embryos is insufficient justification for the conclusion of identity. It is necessary to show histologically that these regenerating structures develop via stages similar to those of zygotic embryogenesis into individual structures with clear bipolarity. The only two publications that present histological evidence of somatic embryogenesis from hypocotyls of *Pelargonium × hortorum* are not convincing. Gill et al.

(1993) showed only a single regenerant classified as a “heart-shaped somatic embryo”, but the structure was connected to the maternal explant over its total width and the transition between the cell types of the explant and the structure was gradual instead of constituting a sharp boundary. Hutchinson et al. (1996a) published the development of these structures more comprehensively, but again the more advanced structures, such as the “cotyledonary-stage embryos”, were broadly linked to the explant, and there was a gradual transition of cell types between the maternal tissue and the structures. These advanced structures showed only a shoot meristem, and not a root pole. Not one bipolar structure was presented.

On the basis of these criticisms of published histological results and the results of the present experiment, we conclude that there has been as yet no proof of somatic embryogenesis from the hypocotyls of *Pelargonium* × *hortorum*, although several papers have already dealt with this matter.

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